Meta-analysis of the predictive value of DNA aneuploidy in malignant transformation of oral potentially malignant disorders

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Abstract

DNA aneuploidy is an imbalance of chromosomal DNA content that has been highlighted as a predictor of biological behavior and risk of malignant transformation. To date, DNA aneuploidy in oral potentially malignant diseases (OPMD) has been shown to correlate strongly with severe dysplasia and high-risk lesions that appeared non-dysplastic can be identified by ploidy analysis. Nevertheless, the prognostic value of DNA aneuploidy in predicting malignant transformation of OPMD remains to be validated. The aim of this meta-analysis was to assess the role of DNA aneuploidy in predicting malignant transformation in OPMD. The questions addressed were 1) Is DNA aneuploidy a useful marker to predict malignant transformation in OPMD? 2) Is DNA diploidy a useful negative marker of malignant transformation in OPMD? These questions were addressed using the PECO method. Five studies assessing aneuploidy as a risk marker of malignant change were pooled into the meta-analysis. Aneuploidy was found to be associated with a 3.12-fold increased risk to progress into cancer (RR = 3.12, 95% CI 1.86-5.24). Based on the 5 studies meta-analyzed, “no malignant progression” was more likely to occur in DNA diploid OPMD by 82% when compared to aneuploidy (RR = 0.18, 95% CI 0.08 – 0.41). In conclusion, aneuploidy is a useful marker of malignant transformation in OPMD, though a diploid result should be interpreted with caution.

Introduction

Oral cancer (oral squamous carcinoma; OC) is a significant burden globally with an annual incidence of 275,000 cases worldwide. The 5-year survival rate for patients with OC is still low in many parts of the world despite significant progress in diagnosis and treatment (1). There is currently no clear evidence to support the consensus that early diagnosis is the most important factor to improve prognosis, as all data available relates to size of the tumour at diagnosis, which is then extrapolated to suggest early diagnosis as a potential marker of good prognosis (1, 2). OC is often preceded by oral potentially malignant disorders (OPMDs), whose risk can be assessed based on the presence and severity of epithelial dysplasia (3). The prevalence of OPMDs has been reported to be approximately 2% worldwide with an overall transformation rate of 1.36% per year (3-5). Lack of consensus on the histological assessment for the presence and degree of dysplasia combined with the fact that not all OPMDs with dysplasia transform to cancer have fueled the search for alternative methods to detect those patients with OPMDs at high risk of developing oral cancer (6, 7).

DNA aneuploidy is an imbalance of chromosomal DNA content that has been highlighted as a predictor of biological behavior and risk of malignant transformation in several types of human tissues (8), such as Barrett's esophagus, ulcerative colitis, colorectal adenomas, melanocytic skin nevi, cervical as well as oral lesions (9-15). DNA content, as evaluated using image cytometry (DNA-ICM) and high-resolution flow cytometry (DNA-FCM) permit the detection of abnormal nuclear DNA content. Both DNA-ICM and DNA-FCM techniques have been considered appropriate for routine analysis in many clinical applications including OPMDs (6, 8).

So far, DNA aneuploidy in OPMDs has been shown to correlate strongly with severe dysplasia (8, 16) and high-risk lesions that appeared non-dysplastic can be identified by ploidy analysis (6, 17). Nevertheless, the prognostic value of DNA aneuploidy in predicting malignant transformation of OPMDs remains to be validated.

The aim of this systematic review and meta-analysis is to assess the role of DNA aneuploidy in predicting malignant transformation in OPMDs.

Methods

Data sources and search strategy

This systematic review was conducted following Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) statement (18). The literature search was conducted using PubMed, EMBASE and Scopus databases.

Searching in those databases was by using a combination of text word and/or Medical Subject Heading (MeSH) terms. The search terms included ("aneuploidy" OR "DNA content") AND ("malignant transformation" OR "oral cancer risk") AND ("oral potentially malignant disorders" OR "oral precancer"). A manual search for the reference list of eligible studies was also carried out for further relevant articles. The grey literature was not searched.

The questions addressed were

1) Is DNA aneuploidy a useful marker to predict malignant transformation in OPMDs?

2) Is DNA diploidy a useful negative marker of malignant transformation in OPMDs?

These questions were addressed using the PECO method.

P (problem): difficulty in predicting lesions that will undergo malignant transformation.

E (exposure): DNA aneuploidy assessment using objective and quantitative methods, namely DNA-ICM and DNA-FCM.

C (comparison) base: DNA diploidy.

O (outcome): positive prediction of malignant change.

Study selection

Eligible studies were selected if they met the following criteria: 1) original cohort studies conducted either retrospectively or prospectively; 2) oral potentially malignant disorders were assessed; 3) DNA ploidy analysis should be investigated as exposure of interest; 4) the primary outcome should be clearly reported as malignant transformation, irrespective of dysplasia grade; 5) they were accessible and published as full papers.

Studies were excluded if the aforementioned outcome was not investigated. Withdrawn/retracted studies, review articles, case-reports, commentaries, letters to the Editor and unpublished articles were excluded. Studies

exclusively investigating high-risk OPMD, such as proliferative leukoplakia (, or exclusively low-risk OPMD, such as lichen planus, were excluded.

Two investigators (NA & SA) independently evaluated the articles retrieved from the databases. They were screened and checked for eligibility criteria. Discrepancies were resolved by a third reviewer (MS).

Data extraction

A standardized data collection form was used for each study and the following data were extracted (whenever available) independently by two investigators: first author's last name for the study, design type (case control or cohort study), number of cases and controls, assessment of exposure and outcome, histopathological grading (mild, moderate, severe or no dysplasia), number of cases and events, hazard risk (HR) estimates for each study with CI 95% and follow-up time.

Statistical analysis

Data analysis was performed by three investigators (DP, MS & NA). As a primary objective of the meta-analysis, we derived the association of aneuploidy with the malignant progression of OPMD by measuring the risk ratio (RRs) with 95 % CI. The association of diploidy with malignant transformation was also evaluated. Review Manager (RevMan) (version 5.3.5; the Nordic Cochrane Centre, Copenhagen, Denmark) was used in this meta-analysis and a p value < 0.05 was considered statistically significant. If there was significant heterogeneity among studies, the random-effects model was used; otherwise, the fixed-effects model was applied. Between-study heterogeneity was examined using the Q-statistic and the I_2 statistic. Funnel plots and qualitative analysis of specific variables of the ploidy analysis method were used to assess publication bias. Furthermore, a sensitivity analysis was performed to examine the robustness of the pooled results, using the leave-one-out approach.

Results

Selection and characteristics of the studies

A total of 611 articles (579 from database, 32 from manual search) were recorded, of which 84 were excluded as duplicate records. After reviewing the titles and abstracts, 402 records were excluded. Secondary screening yielded 23 articles to be evaluated for eligibility. Fourteen studies were further excluded based on the inclusion and exclusion criteria and 3 final studies were excluded as they either exclusively investigated cases of proliferative verrucous leukoplakia (19, 20) or exclusively lichen planus (17). Finally, DNA ploidy analysis for malignant progression of OPMD was assessed in 5 studies, totalizing 528 cases (Fig. 1 and Table 1) (16, 21-24). Out of 5 studies, 2 included all types of OPMD, two included dysplastic lesions and 1 included leukoplakia [table 1]. In all eligible studies, DNA ploidy analysis was carried out on formalin-fixed paraffin-embedded (FFPE) tissues using image-based cytometry.

The studies by Sperandio et al. 2013 (16), Bradley et al. 2010 (21) and Siebers et al. 2013 (23), though retrospective, were designed to investigate a cohort of OPMD lesions in chronological order of presentation and biopsy over a period of 10 years and minimal follow-up of 5 years thereafter. They compared the outcome of ploidy-analyzed patients with OPMD against those from otherwise innocuous lesions, such as fibroepithelial polyps and squamous cell papillomas, which were all diploid. No malignant transformation was detected in such control lesions. Careful follow-up for malignant transformation was ascertained against national cancer registry databases for the respective countries in which the studies were performed. The remaining 2 studies (Bremmer et al. 2011 and Torres-Rendon et al. 2009) (22, 24) were retrospective case-controls, focusing on dysplastic lesions.

Quantitative synthesis

Meta-analysis was performed on the 5 shortlisted studies for systematic review.

DNA aneuploidy and malignant progression of OPMD

Five studies assessing aneuploidy as a risk marker of malignant change were pooled into the meta-analysis. As shown in Fig. 2 aneuploidy is associated with a 3.12-fold increased risk to progress into cancer (RR = 3.12, 95%

CI 1.86-5.24). Moderate heterogeneity was observed (I_2 =65%; p=0.02), though heterogeneity dropped to zero if the study by Bremmer et al. 2011 was disregarded (table 2).

DNA diploidy and no malignant progression

Based on the 5 studies meta-analyzed, "no malignant progression" was more likely to occur in DNA diploid OPMD by 82% when compared to aneuploidy (RR = 0.18, 95% CI 0.08 – 0.41), as illustrated in Fig. 3. High heterogeneity was observed in this analysis (I_2 = 91%; p<0.00001), though, again, removing the study by Bremmer et al. 2011, heterogeneity dropped to moderate (I_2 = 49%; p<0.12) (table 3).

Sensitivity analysis

Influence analyses using the leave-one-out approach were performed for the meta-analyses to evaluate the stability of the RR and heterogeneity (tables 2 and 3). These analyses showed that the corresponding RR were not significantly altered, demonstrating confidence that the overall meta-analyses were robust, despite the moderate to high heterogeneity detected.

Publication bias

The funnel plots analysis presented in Figs. 4 A & B indicated a low likelihood of publication bias. An itemized assessment of specific variables of the DNA ploidy methodology itself was performed to further validate the funnel plots. Such variables included: case selection, nuclei isolation and monolayer staining method, image-capture hardware and software (whether automated or manual), image editing (to eliminate overlapping and damaged nuclei as well as debris), endogenous control and ploidy classification criteria, including CV values. From a qualitative viewpoint, the studies were found to be considerably similar in their description of such variables. All 5 studies reviewed herein adhered strictly to previously published criteria (25). Tissue preparation was based on 2 to 6 50μm tissue sections from FFPE biopsies, enzymatically digested to yield a suspension of cell nuclei, from which monolayers were produced and stained using the Feulgen method. All authors reported using an automated image-capture system, which also allowed manual fine-tune editing *post hoc*. Lymphocytes were used as an internal control to ascertain the location of the diploid peak in all studies and the ploidy

classification criteria were also the same for all articles and were based on the guidelines by the European Society for Analytical Cellular Pathology (25). All authors reported a CV < 5 for the diploid peak.

Discussion

DNA content analysis has been proposed as a good prognostic marker of OPMD, in which an aneuploid diagnosis was associated with a higher risk of malignant transformation than a diploid case. Such method proposed to resolve the issue of subjectivity involved in the process of histological grading of epithelial dysplasia. Nevertheless, no attempt has been made to systematically review and/or meta-analyze the current evidence on DNA ploidy analysis as a prognostic marker of such lesions. The present study brings new insight into the usefulness of ploidy analysis as a prognostic marker in the management of early potentially malignant lesions.

The first meta-analysis demonstrated that aneuploidy increased the risk of malignant transformation (RR= 3.12). The leave-one-out sensitivity analysis proved this finding robust, which reinforces the ability of DNA aneuploidy to identify high-risk lesions, as established by image-cytometry, as previously proposed (16, 21-24).

Whilst an aneuploid result may mean a high risk of malignant change, a diploid outcome does not necessarily mean no risk at all. The second meta-analysis revealed that a diploid result carries approximately 80% risk of not undergoing malignant transformation (figure 3), though 20% of cases may still be at risk of transforming. The most likely explanations for such a limitation are sampling error of the affected mucosa at biopsy, which risks false negative results; and limitation of the DNA ploidy method itself, which may not be sensitive enough to detect minor degrees of chromosomal instability on a single sample (26). This may occur due to the nuclei being isolated from thick sections of paraffin blocks without prior microdissection of the specimen, which may ‘dilute out’ the aneuploid cells and compromise the sensitivity of the test.

The advantage of ploidy analysis over the traditional method of qualitative dysplasia grading is objectivity. Identifying and scoring epithelial dysplasia demands an experienced and trained pathologist, which is costly and of limited availability in many health systems, especially in developing areas of the world. Such an approach also suffers from subjectivity, as many studies have shown considerable variation in agreement levels both inter and intra-examiner (27-31). Ploidy analysis is regarded as an objective method, based on automated image analysis and well-established strict threshold criteria for ploidy classification (25). This provides evidence that this study brings added value to the current knowledge on objective approaches to manage OPMD.

The reviewed studies were all conducted in developed countries, which may present a lower incidence of some risk factors for the development of oral cancer, such as nutritional deficiencies and low socioeconomic status, although tobacco (either smoked or chewed) and alcohol drinking remain the most important risk factors

anywhere in the world. Furthermore, chromosomal instability is considered a fundamental process common to all cancers (32). Therefore, it is likely that such evidence could be applicable to developing countries; especially as DNA ploidy analysis detects alterations at the nuclear content level, regardless of the initiating factors. Nevertheless, further studies are needed to validate this assumption in the developing world. Furthermore, an objective test such as DNA ploidy analysis might prove more cost-effective in the long run when compared to histological grading, due to scarcity of specialized human resources, as discussed elsewhere. This could in turn favour the underprivileged areas of the world.

The final number of articles shortlisted for this review and meta-analysis was relatively low (N=5) due mainly to the overall scarcity of articles published using ploidy analysis to prognosticate OPMD. Furthermore, the strict selection criteria used herein meant that no flow cytometry studies could be included. For example, previous studies using flow cytometry preferably tested the association between dysplasia grades (33) or copy number aberrations (34) and ploidy abnormalities without malignant transformation as an outcome. Despite an outcome variable clearly defined as malignant transformation, the study by Giaretti et al. 2013 used DNA index as the exposure test, which is only part of the criteria for the diagnosis of aneuploidy (25). Therefore, these studies were excluded.

Despite differences in study design, namely cohort *vs.* case-control, very similar outcome in terms of malignant transformation rates was observed across the studies. The risk of population sampling bias based on study design was therefore very low. This was further demonstrated using the leave-one-out sensitivity analyses on the data, whereby removal of the study by Bremmer et al. 2011 considerably lowered the heterogeneity score (I_2, table 2) without significantly influencing the RR. Funnel plots and quality assessment of method description reinforced the low risk of publication bias.

Despite the low risk of publication bias suggested by the funnel plots, publication bias was further assessed on specific parameters of the methods described within the articles, which were based on published guidelines (25, 26) and were uniformly reported across the studies, which validates the funnel plots, thus reassuring the low risk of publication bias.

Based on the analysis performed herein, the authors conclude that aneuploidy is a useful marker of malignant transformation in OPMD but that a diploid result should be interpreted with caution. Further studies are needed to refine the DNA ploidy analysis test in order to improve positive and negative predictive values.

References

1. Arduino PG, Carrozzo M, Chiecchio A et al. Clinical and histopathologic independent prognostic factors in oral squamous cell carcinoma: a retrospective study of 334 cases. J Oral Maxillofac Surg 2008; 66: 1570-9.

2. Kademani D, Bell RB, Bagheri S et al. Prognostic factors in intraoral squamous cell carcinoma: the influence of histologic grade. J Oral Maxillofac Surg 2005; 63: 1599-605.

3. Mehanna HM, Rattay T, Smith J et al. Treatment and follow-up of oral dysplasia - a systematic review and meta-analysis. Head Neck 2009; 31: 1600-9.

4. Petti S. Pooled estimate of world leukoplakia prevalence: a systematic review. Oral Oncol 2003; 39: 770-80.

5. Warnakulasuriya S, Johnson NW, van der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med 2007; 36: 575-80.

6. Dionne KR, Warnakulasuriya S, Zain RB et al. Potentially malignant disorders of the oral cavity: current practice and future directions in the clinic and laboratory. Int J Cancer 2015; 136: 503-15.

7. Pitiyage G, Tilakaratne WM, Tavassoli M et al. Molecular markers in oral epithelial dysplasia: review. J Oral Pathol Med 2009; 38: 737-52.

8. Giaretti W, Pentenero M, Gandolfo S et al. Chromosomal instability, aneuploidy and routine high-resolution DNA content analysis in oral cancer risk evaluation. Future Oncol 2012; 8: 1257-71.

9. Giaretti W. A model of DNA aneuploidization and evolution in colorectal cancer. Lab Invest 1994; 71: 904-10.

10. Lavelle CL, Scully C. Criteria to rationalize population screening to control oral cancer. Oral Oncol 2005; 41: 11-6.

11. Nankivell P, Mehanna H. Oral dysplasia: biomarkers, treatment, and follow-up. Curr Oncol Rep 2011; 13: 145-52.

12. Newton JA, Camplejohn RS, Mcgibbon DH. The flow cytometry of melanocytic skin lesions. Br J Cancer 1988; 58: 606-9.

13. Rabinovitch PS, Dziadon S, Brentnall TA et al. Pancolonic chromosomal instability precedes dysplasia and cancer in ulcerative colitis. Cancer Res 1999; 59: 5148-53.

14. Reid BJ, Levine DS, Longton G et al. Predictors of progression to cancer in Barrett's esophagus: baseline histology and flow cytometry identify low- and high-risk patient subsets. Am J Gastroenterol 2000; 95: 1669-76.

15. Risques RA, Lai LA, Brentnall TA et al. Ulcerative colitis is a disease of accelerated colon aging: evidence from telomere attrition and DNA damage. Gastroenterology 2008; 135: 410-8.

16. Sperandio M, Brown AL, Lock C et al. Predictive value of dysplasia grading and DNA ploidy in malignant transformation of oral potentially malignant disorders. Cancer Prev Res 2013; 6: 822-31.

17. Sperandio M, Klinikowski MF, Brown AL et al. Image-based DNA ploidy analysis aids prediction of malignant transformation in oral lichen planus. Oral Surg Oral Med Oral Pathol Oral Radiol 2016; 121: 643-50.

18. Moher D, Liberati A, Tetzlaff J et al. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med 2009; 6: e1000097.

19. Gouvea AF, Santos Silva AR, Speight PM et al. High incidence of DNA ploidy abnormalities and increased Mcm2 expression may predict malignant change in oral proliferative verrucous leukoplakia . Histopathology 2013; 62: 551-62.

20. Klanrit P, Sperandio M, Brown AL et al. DNA ploidy in proliferative verrucous leukoplakia. Oral Oncol 2007; 43: 310-6.

21. Bradley G, Odell EW, Raphael S et al. Abnormal DNA content in oral epithelial dysplasia is associated with increased risk of progression to carcinoma. Br J Cancer 2010; 103: 1432-42.

22. Bremmer JF, Brakenhoff RH, Broeckaert MA et al. Prognostic value of DNA ploidy status in patients with oral leukoplakia. Oral Oncol 2011; 47 : 659 : 960

23. Siebers TJ, Bergshoeff VE, Otte-Holler I et al. Chromosome instability predicts the progression of premalignant oral lesions. Oral Oncol 2013; 49: 1121-8.

24. Torres-Rendon A, Stewart R, Craig GT et al. DNA ploidy analysis by image cytometry helps to identify oral epithelial dysplasias with a high risk of malignant progression. Oral Oncol 2009; 45: 468-73.

25. Haroske G, Baak JP, Danielsen H et al. Fourth updated ESACP consensus report on diagnostic DNA image cytometry. Anal cell pathol 2001; 23: 89-95.

26. Diwakar N, Sperandio M, Sherriff M et al. Heterogeneity, histological features and DNA ploidy in oral carcinoma by image-based analysis. Oral Oncol 2005; 41: 416-22.

27. Abbey LM, Kaugars GE, Gunsolley JC et al. Intraexaminer and interexaminer reliability in the diagnosis of oral epithelial dysplasia. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1995; 80: 188-91.

28. Fleskens S, Slootweg P. Grading systems in head and neck dysplasia: their prognostic value, weaknesses and utility. Head Neck Oncol 2009; 1: 11.

29. Karabulut A, Reibel J, Therkildsen MH et al. Observer variability in the histologic assessment of oral premalignant lesions. J Oral Pathol Med 1995; 24: 198-200.

30. Kujan O, Khattab A, Oliver RJ et al. Why oral histopathology suffers inter-observer variability on grading oral epithelial dysplasia: an attempt to understand the sources of variation. Oral Oncol 2007; 43: 224-31.

31. Pindborg JJ, Reibel J, Holmstrup P. Subjectivity in evaluating oral epithelial dysplasia, carcinoma in situ and initial carcinoma. J Oral Pathol 1985; 14: 698-708.

32. Potapova TA, Zhu J, Li R. Aneuploidy and chromosomal instability: a vicious cycle driving cellular evolution and cancer genome chaos. Cancer Metastasis Rev 2013; 32: 733-389

33. Van Zyl AW, Van Heerden MB, Langenegger E et al. Correlation between dysplasia and ploidy status in oral leukoplakia. Head Neck Pathol 2012; 6: 322-7.

34. Castagnola P, Zoppoli G, Gandolfo S et al. Genomic DNA Copy Number Aberrations, Histological Diagnosis, Oral Subsite and Aneuploidy in OPMDs/OSCCs. PloS One 2015; 10: e0142294.

35. Barnes L, Eveson JW, Reichart P, Sidransky D. World Health Organization classification of tumours: pathology and genetics of head and neck tumours. Lyon: IARC Press; 2005. p. 140–3.

36. Warnakulasuriya S, Reibel J, Bouquot J, Dabelstein E. Oral dysplasia classification systems: predictive value, utility, weaknesses and scope for classification systems: predictive value, utility, weaknesses and scope for improvement. J Oral Pathol Med 2008; 37:127–33.

Figure and Table Legends:

Table 1. Summary of studies included in the meta-analysis.

Table 2. Leave-one-out sensitivity test for the meta-analysis on the risk ratio (RR) of malignant transformation of DNA aneuploid lesions according to the 5 studies selected.

Table 3. Leave-one-out sensitivity test for the meta-analysis on the risk ratio (RR) of no malignant transformation between DNA diploid and DNA aneuploid lesions, according to the 5 studies selected.

Figure 1. PRISMA (Preferred Reporting Items for Systematic Reviews) flow diagram for the systematic review.

Figure 2. Forest plot of studies evaluating Risk Ratio (RR) of DNA aneuploidy for malignant transformation of OPMD

Figure 3. Forest plot of studies evaluating Risk Ratio (RRs) of NO malignant transformation of diploid and aneuploid OPMD

Figure 4. A Funnel plot analysis for association of DNA ploidy with malignant transformation of OPMD. B Funnel plot analysis for association of ploidy with NO malignant transformation.

Table 1

Study, Year	Study design	Cases	Control	Progressed	Not progressed	Histopathologic Grading n (Aneuploidy)	HR	Follow-up
				n (Aneuploidy)	n (Aneuploidy)			
Torres-Rendon et al. 2009	Retrospective case control	42	44	42 (14)	44 (5)	None 0 (0) Mild 4 (0) Moderate 25 (8) Severe 57 (11)	NA	Mean (55.8 ± 8.0) Mean (C) (59.17±15.76)
Bradley et al 2010	Retrospective case control	49	50	49 (22)	50 (6)	None 0 (0) Mild 50 (7) Moderate 34 (13) Severe 15 (8)	3.3 (95%CI: 1.5-7.4) $p = 0.003$	Range (6-131)
Bremmer et al 2011	Prospective cohort	62	0	13 (7)	49 (20)	None 31 (18) Mild 18 (11) Moderate 72 (42) Severe 4 (2)	3.7 (CI: 1.1-13.0) $P = 0.04$	Median 69 mons. (range 10-193)
Sperandio et al 2013	Retrospective cohort	273	80	32 (20)	241 (39)	None 31 (18) Mild 18 (11) Moderate 72 (42) Severe 49 (29)	7.4 (95% CI: 3.4-15.9) $P < 0.001$	Minimum 5 Years
Siebers et al 2013	Retrospective	102	0	16 (10)	86 (13)	None 66 (7) Mild 16 (5) Moderate 17 (8) Severe 3 (3)	7.2 (CI: 2.61-20.03) $P < 0.001$	Median: 27.0
Sperandio et al 2016	Retrospective case control	14	42	21 (4)	41 (0)	NA	NA	14yrs. (10-18yrs)

NA: not available

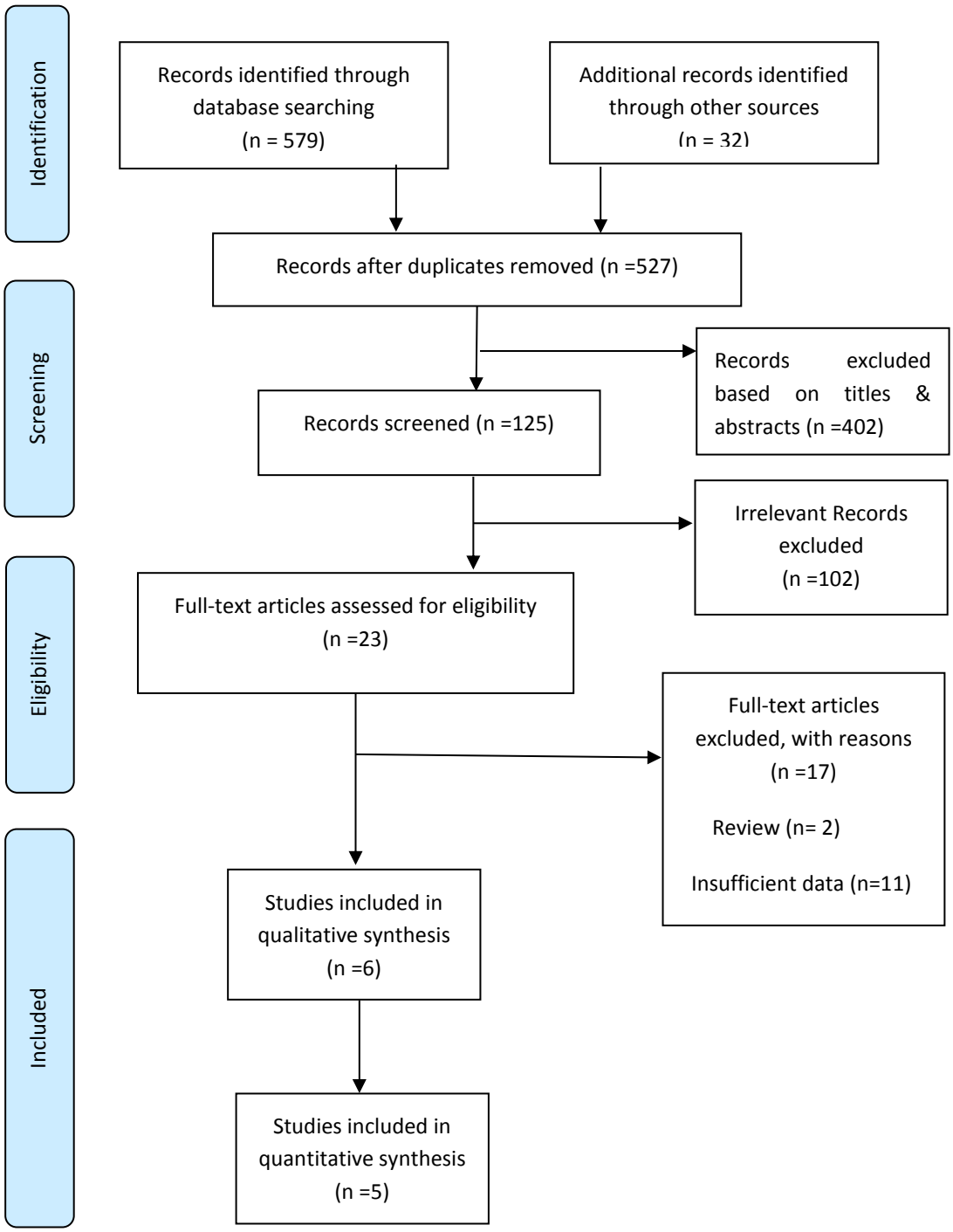
Table 2

Removed	RR	Heterogeneity (I^2 - %)	*p* value (I^2)	Z value	*p* value (Z)
None	3.12	65	0.02	4.32	< 0.0001
Torres-Rendon et al. 2009	3.18	74	0.01	3.62	0.0003
Bradley et al. 2010	3.02	73	0.01	3.37	0.0007
Bremmer et al. 2011	3.95	0	0.7	8.66	< 0.0001
Sperandio et al. 2013	2.94	69	0.02	2.94	0.003
Siebers et al. 2013	2.73	66	0.03	3.52	0.0004
Mean	3.16	57.83		4.41	
SD	0.42	28.56		2.13	

Table 3

Removed	RR	Heterogeneity (I^2 - %)	*p* value (I^2)	Z value	*p* value (Z)
None	0.18	91	< 0.0001	4.13	< 0.0001
Torres-Rendon et al. 2009	0.20	93	< 0.0001	3.40	0.0007
Bradley et al. 2010	0.20	93	< 0.0001	3.36	0.0008
Bremmer et al. 2011	0.14	49	0.12	8.82	< 0.0001
Sperandio et al. 2013	0.18	93	< 0.0001	2.63	0.009
Siebers et al. 2013	0.23	91	< 0.0001	3.44	0.0006
Mean	0.19	85.00		4.30	
SD	0.03	17.66		2.27	

Figure 1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Irrelevant Records excluded (n =102)
Full-text articles assessed for eligibility (n =23)
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Figure 2

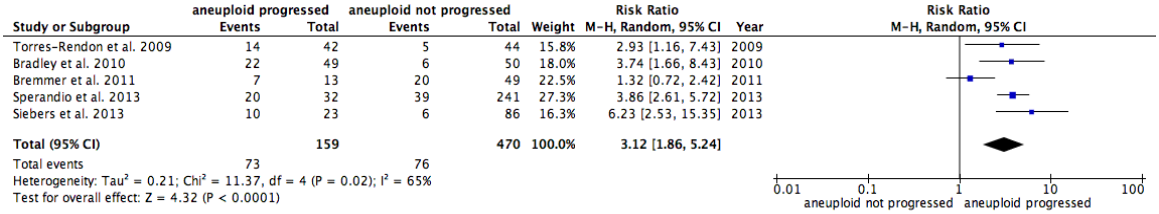


Figure 3

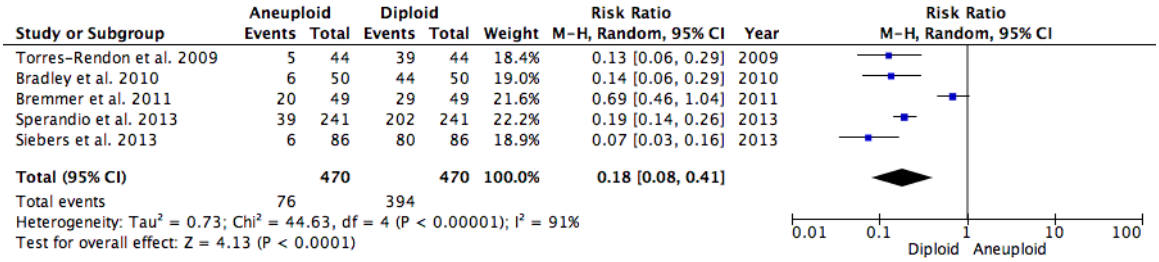
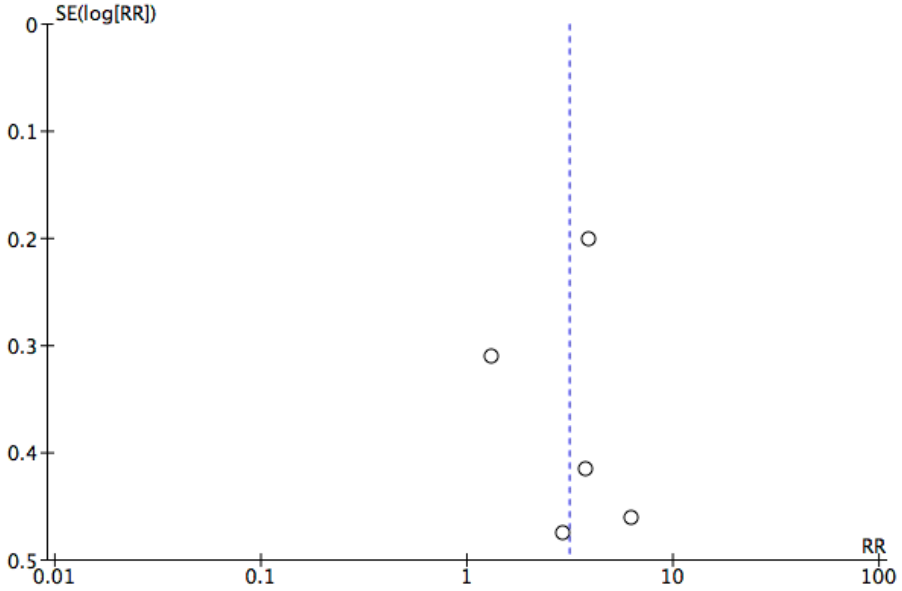


Figure 4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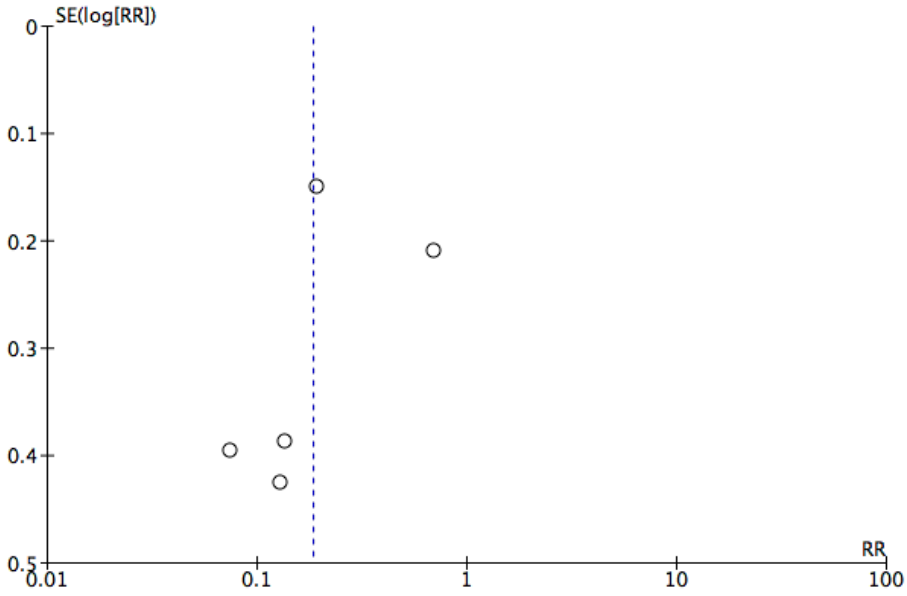
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